CHAPTER-3

Determination of toxic heavy metals in Cholic acid using quadrupole inductively coupled plasma mass spectrometry (Q-ICP-MS)

3.1: INTRODUCTION

One of the primary bile acids is called Cholic acid. Bile acids are biological substances that are a part of the steroidal family. They are produced in humans (by the liver), as well as in the livers of the majority of other animals [1,2]. A diet high in lipids may be the cause of a buildup of hazardous substances in the liver, such as Zn, Co, Cd, Mn, and Ni. The liver is in charge of making bile acids. Inhibition of enzymatic activity and change of metabolic pathways are both outcomes of an accumulation of toxic heavy metals in the liver, which causes the accumulation. The presence of such dangerous toxic heavy metals also increases the risk of developing a cancer, according to studies [3].

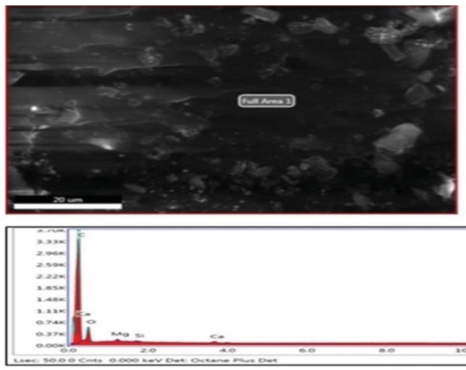
The medical monitoring and quantification of potentially toxic heavy metals in the liver or in liver byproducts such bile acids are essential. This is the case whether the metals are found in the liver or the liver's byproducts. Although a search on Web of Science using the terms Cholic acid and harmful heavy metals yields four items, none of them are relevant to either the purpose of the present investigation or the findings of it. The purpose of this research is to collect all of the data possible on the levels of hazardous metals that are present in cholesterin-containing medications that are taken by people on a regular basis and to determine whether or not those levels exceed the maximum allowable level specified by the United States Pharmacopoeia (USP) 233 standard.

Not much of an introduction is needed to explain that V, Ni, Cd, Hg, Pb, and As are all toxic heavy metals that can be found in water, food, drugs, and the environment and that their presence has negative consequences [4–12]. The agencies tasked with policing the pharmaceutical business have set limits on how much heavy metals can be included in pharmaceuticals. Regular limit checks ensure that these peak values are being met. By doing these analyses, we can be sure that no inorganic pollutants were added to the medications during production. Total metal impurity concentrations in pharmaceutical goods are being monitored by a collaborative effort by the United States Pharmacopoeia (USP), British Pharmacopoeia (BP), European Pharmacopoeia (EP), and Japanese Pharmacopoeia (JP). However, the currently used techniques are not particularly specific, not particularly sensitive, and not particularly quick. The few recent laws, such as USP 232 and 233 are the sole exceptions to this rule. Therefore, very sensitive and selective approaches are urgently required for identifying trace dangerous heavy metals in pharmaceutical compounds. This is

important not only had to ensure the safety and efficacy of drugs designed for human use, but also to meet the demanding regulatory criteria [13].

Since plasma allows ionisation to occur in a chemically inert environment, preventing oxide formation, and the ionisation is more complete than with other energy sources like flame ionisation, it is advantageous to use plasma as the energy source in quadrupole inductively coupled plasma mass spectrometry (Q-ICP-MS). Q-ICP-MS analysis of dangerous heavy metals is superior to other approaches including atomic absorption spectrometry, X-ray fluorescence spectrometry, and ICP optical emission spectrometry due to its exceptionally low detection limits for a wide variety of elements. Toxic heavy metals are analysed using these alternative techniques. [14] It is possible to measure the size of some components to within a billionth of a trillionth of a trillionth of an inch. Several researchers have used the cutting-edge analytical technique of Q-ICP-MS for bio analytical purposes in the past [15–17].

ICP-OES analysis was undertaken in addition to Q-ICP-MS analysis of the dangerous heavy metals detected in Cholic acid for the goal of creating a comparison. In addition, SEM-EDAX was used to perform a comprehensive characterization of a commercial sample of Cholic acid to establish its purity (Fig. 1).



Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	R	A	F
C K	78.44	83.21	1,377.48	3.72	0.60	1.01	1	0.76	1
O K	20.56	16.37	138.58	11.57	0.03	0.96	1.01	0.13	1
MgK	0.40	0.21	22.33	23.76	0.00	0.89	1.04	0.63	1
SiK	0.14	0.06	10.20	57.64	0.00	0.87	1.05	0.88	1
CaK	0.47	0.15	19.96	31.12	0.00	0.82	1.08	1.04	1

Fig. 1: SEM-EDAX analysis of Cholic acid (commercial sample)

In order to accurately determine the aforementioned dangerous heavy metal contaminants in oral pharmaceutical goods with a single test, the goal of this work is to develop a quick, efficient, easy-to-understand, and accurate method of Cholic acid sample preparation that can be used in conjunction with Q-ICP-MS.

3.2: RESULTS AND DISCUSSION

3.2.1: Internal benchmark for the analysis of potentially hazardous heavy metals

When conducting elemental analysis using Q-ICP-MS, establishing an acceptable internal standard is one of the most important steps. This would have a major effect on the reliability of the findings.

3.2.2: Optimisation of the Working Parameters of the Q-ICP-MS Instrument

Several sets of optimised Q-ICP-MS parameters have been reported.

3.2.3: Method validation

In the field of analytical chemistry, one of the technical parts that make up the entire quality assurance scheme is method validation. The element that is used and the potential interferences that can be caused by it are what define selectivity and specificity. It is always determined by "the extent to which the approach may be employed to determine the specific analytes in mixtures or matrices without interference from other components that behave similarly [18]." Primary isotopes of each element were used to examine the selectivity of the current method: ^{51}V, ^{59}Co, ^{60}Ni, ^{111}Cd, ^{202}Hg, ^{208}Pb, and ^{75}As. Evaluation and proof of the procedure's dependability were the goals of the validation study.

3.2.4: Estimated LOD

Lowest detectable concentration (LOD) of an analyte in a sample; nevertheless, LOD may not always equate to quantifiable concentration. It is referred to as a limit test, and its purpose is to detect whether or not an analyte is above or below a particular threshold based on the calibration function using Equation (1) [19].

$$\text{LOD} = \frac{3.3\,\sigma}{\text{S}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Where; denotes the average deviation from the mean.

The slope, denoted by S, is calculated using the calibration curve.

The calculated LODs for the elements V, Co, Ni, Cd, Hg, Pd, and As were found to be 0.01, 0.01, 0.18, 0.002, 0.02, 0.02, and 0.10 g/L, respectively. Minimum practical amounts of the elements evaluated in the samples were determined by analysing three replicates at 30 g/L for V and 15 g/L for Co. The accuracy of these concentration calculations is sufficient. The predicted LODs for the elements V, Co, Ni, Cd, Hg, Pd, and as were found to be 0.01, 0.01, 0.18, 0.002, 0.02, 0.02, and 0.10 g/L, respectively. Analysis using three replicates at the following concentrations determined the minimal feasible amounts of the elements tested in the samples that can be determined with an acceptable level of accuracy: The results were shown in Table 4 at concentrations of 30 g/L for V, 15 g/L for Co, 60 g/L for Ni, and 1.5 g/L for.

3.2.5: Estimated LOQ

The LOQ is defined as the concentration of an analyte in a sample below which the method can be reliably and accurately performed under the stated operating circumstances. For LOQ, the noise-to-signal ratio must be no less than 1:10. V, Co, Ni, Cd, Hg, Pb, and as all had estimated LOQs of between 30 and 15 mg/L, while Cd, Hg, Pb, and as all had LOQs of between 1.5 and 9.0 mg/L. The combined results are shown in Table 4. [20].

An analytical technique known as e Q-ICP-MS has its parameters listed in Table 3. These parameters are crucial for controlling and optimizing the performance of the analytical instrument, ensuring accurate and precise measurements of elements within a sample. The RF power is set at 1600 W, which plays a critical role in generating high-temperature plasma required for ionizing sample components. RF matching is set at 1.80 V, ensuring efficient power transfer to the plasma. The sampling depth is set at 4.6 mm, defining the position within the plasma where the sample aerosol is introduced. The carrier gas flow rate is set at 1.02 L per minute, ensuring consistent and stable sample introduction. The spray chamber temperature is maintained at 2 °C, influencing the condensation and desolvation of the sample aerosol. The extract voltage is set at 3.7 V, controlling the movement of ions out of the quadrupole mass analyzer. The Einzel 1 and Einzel 3 V are set at -100 V and 22 V,

respectively, influencing ion transmission. Cell entrance and exit voltages are set at -50 V, -42 V, and -43 V, respectively. Plate bias voltage is set at -43 V, Q Pb bias voltage at -4.6 V, OctPc RF voltage at 190 V, and OctP bias voltage at -7.0 V. Q-ICP-MS is a potent technology for trace element analysis across many disciplines thanks to the meticulous optimisation of its parameters, which guarantees precise and accurate measurements of elements in samples.

Table 3: Typical Q-ICP-MS instrument parameters for the analytical method

Parameter	Setting
RF[a] power (W)	1600
RF matching (V)	1.80
Sampling depth (mm)	4.6
Carrier gas (L min^{-1})	1.02
Spray chamber temperature (°C)	2
Nebulizer pump (revolutions per second, rps)	0.1
Extract (V)	3.7
Einzel 1,3 (V)	−100
Einzel 2 (V)	22
Cell entrance (V)	−50
Cell exit (V)	−42
Plate bias (V)	−43
QP[b] bias (V)	−4.6
OctP[c] RF (V)	190
OctP bias (V)	−7.0

[a]RF: Radiofrequency; [b]QP: Quadrupole; [c]OctP: Octupole

3.2.6: Method linearity

Linearity is defined as the ability of a testing technique to generate results that are directly proportional to the concentration of analyte in the sample, according to the CPMP [21] criteria. This ability must be proven within a specified range. The analytical response is linear over the range of concentrations provided, yielding accurate quantification results, if the coefficient of determination (R2) of the calibration curve is greater than 0.995. The linearity of the approach was examined across a predetermined operating range using reference standards of varying concentrations.

Conformity of the calibration curves to a linear relationship

It was discovered that the dynamic linear range is linear from to 30 to 150 µg/L for ^{51}V, 15–75 µg/L for ^{59}Co, 60–300 µg/L for ^{60}Ni, 1.5–7.5 µg/L for ^{111}Cd, 9–45 µg/L for ^{202}Hg and 1.5–12.5 ^{208}Pb and 4.5–22.5 µg/L for ^{75}As (Fig. 1) for Sample 1.

The linearity of the method

The linearity of the approach was evaluated utilising seven distinct levels of samples at each 0, 30, 50, 100, 150, 200, and 250 µg/L for ^{51}V, 0, 15, 25, 50, 75, 100, 125 µg/L for ^{59}Co, 0, 60, 100, 200, 300, 400, 500 µg/L for ^{60}Ni, 0, 1.5, 2.5, 5, 7.5, 10, 12.5 µg/L for ^{111}Cd, ^{208}Pb, 0, 9, 15, 30, 45, 60, 75 µg/L for ^{202}Hg, and 0, 4.5, 7.5, 15, 22.5, 30, 37.5 µg/L for ^{75}As. The method linearity was found to be linear from LOQ values up to 30, 15, 60, 1.5, 9, 1.5, and 4.5 µg/L for ^{51}V, ^{59}Co, ^{60}Ni, ^{111}Cd, ^{202}Hg, ^{208}Pb, and ^{75}As (Fig. 2) for Sample-1.

3.2.7: Method accuracy

According to the principles established by the ICH, accuracy in the context of an analytical procedure "is sometimes termed as trueness." To what extent an analytical procedure agrees with a value that is generally accepted as true or as acceptable determines how accurate the procedure is. For seven elements in Sample-1, Table 4 provides estimates for the LOD, LOQ, and maximum allowable limits. Analytical chemistry relies heavily on these metrics, which evaluate the precision and security of analytical measurements. Vanadium (V), cobalt (Co), nickel (Ni), cadmium (Cd), mercury (Hg), lead (Pb), and arsenic (As) are the seven elements listed in the table. For any given analyte, the LOD (Limit of Detection) is the lowest

concentration at which it may be reliably detected, if not quantified. The practical LOQ is the lowest concentration of an analyte that can be both detected and accurately quantified. Variation in elemental content within a sample is quantified by its CV% (Coefficient of Variation). The maximum permissible limits (µg/L) indicate the maximum allowable concentration of each element as per Egyptian, European Union (EU), and World Health Organization (WHO) standards. These data help determine the reliability and precision of the analytical method used for these measurements and whether the concentrations pose any potential health or environmental risks.

Table 4: Sample-1 estimated limits of detection, practical limits of quantification, and maximum allowable limits (number of replicates=6)

Element	Estimated values		Practical values		CV %	Maximum permissible limits (µg/L)		
	Standard deviation (SD)	LOD (µg/L)	LOQ (µg/L)	Mean concentration ± SD		Egyptian	EU	WHO
V	0.004482	0.01346	30	30.9±0.32	1.03	-	-	-
Co	0.003981	0.005803	15	15.2±0.29	1.90	-	-	-
Ni	0.03359	0.1795	60	59.8±1.31	2.19	20	20	70
Cd	0.003963	0.001525	1.5	1.6±0.07	4.57	3	5	3
Hg	0.004465	0.02271	9.0	9.7±0.35	3.61	1	1	6
Pb	0.004448	0.02066	1.5	1.5±0.01	0.51	10	10	10
As	0.008723	0.09737	4.5	4.7±0.32	6.86	10	10	10

LOQ: Limit of quantification,

LOD: Limit of detection

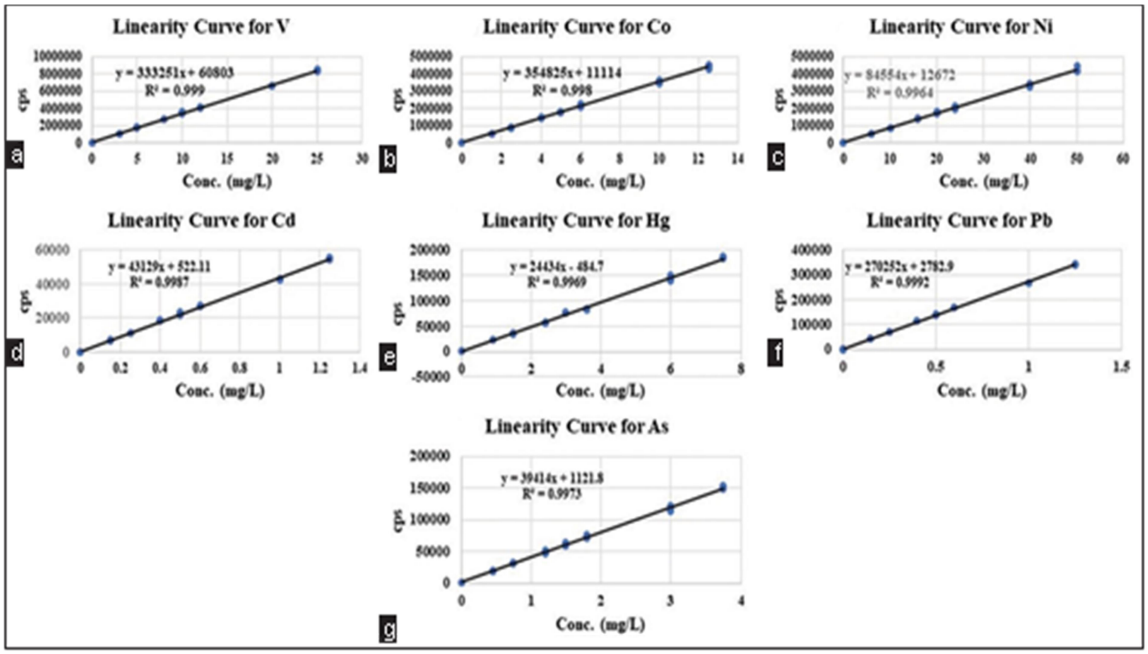


Fig. 2: V (3.0-25.0 mg/L), Co (1.5-12.5 mg/L), Ni (6.0-50.0 mg/L), Cd (0.15-0.25 mg/L), Hg (0.9-7.5 mg/L), Pb (0.15-0.25 mg/L), and As (0.45-0.75 mg/L) linearity of the method.)

Reference value in contrast to the observed value. Since each of these aspects affects the final output in some way, accuracy is best understood as a combination of trueness and precision [22]. Accuracy can be assessed by analysing a sample using the "method to be validated" [23] after adding a known concentration of analyte standard to the sample matrix.

This meticulous study places an emphasis on the ability to be repeated and reproduced.

When applied repeatedly to multiple samples, a method's precision is measured by how well the results of each test agree with one another. The International Conference on Harmonisation (ICH) recognises three separate levels of accuracy: repeatability, intermediate precision, and reproducibility. The RSD precision was computed using Equation 2, and a unified estimate of the precision uncertainty was determined using Equation 3.

RSD = (S* 100)/x……..(2)

Table 5: Linear regression analysis for Sample-1

Element	Linear range (mg/L)	Slope	Intercept	Determination coefficient
V	0.3–25.0	333251	60803	0.9990
Co	1.5–12.5	354825	11114	0.9980
Ni	6.0–50.0	84554	12672	0.9964
Cd	0.15–1.25	43129	522.11	0.9987
Hg	0.9–7.5	24434	484.70	0.9969
Pb	0.15–1.25	270252	2783	0.9992
As	0.45–3.75	39414	1122	0.9973

Table 5 presents the linear regression analysis results for various elements in Sample-1, including the linear range, slope, intercept, and determination coefficient (R^2). The linear range indicates the concentration range within which the instrument or analytical method is reliable for quantification. The slope represents the rate at which the analytical signal changes concerning changes in the element's concentration. A higher slope indicates a more pronounced response to concentration changes. The intercept represents the signal when the linear regression line crosses the y-axis, indicating the instrument's sensitivity to each element. How well the linear regression line fits the data is quantified by the determination coefficient (R2), which can take on values between 0 and 1, with larger values indicating a better fit. The high R^2 values observed (e.g., 0.9990 for vanadium) suggest that the linear regression analysis provides an excellent fit to the data, indicating a strong correlation between concentration and signal. These results are valuable for quality control and ensuring the precision of the analytical method used.

Where,

RSD = Relative standard deviation S = Standard deviation

x = Mean of the data

$$\text{Uncertainty (u)} = \sqrt{\left[\sum (x_{i-\mu}) 2/(n^{*}(n-1))\right]} \quad \ldots\ldots\ldots\ldots\ldots \quad (3)$$

Where,

x_i = i^{th} reading in the data set

μ = Mean of the data set

n = Number of readings in the data set

The results of the repeatability test, which were stated as RSD, indicated that for 111Cd, 202Hg, 208Pb, 75As, 51V, and 59Co, respectively, the RSD was 2.74%, 2.46%, 1.95%, 5.02%, 3.45%, 2.56%, and 2.64%. For 60Ni, the RSD was 2.64%. Table 5 presents the findings of the linear regression analysis that was performed.

An estimation of the amount of measurement error

To the EURACHEM/CITAC GUIDE CG4, "uncertainty" (of measurement) is "A parameter associated with the result of a measurement that characterises the dispersion of the values that could reasonably be attributed to the measurand." There are several factors to consider, including as the accuracy of the laboratory's scales, the thoroughness with which samples and standards are prepared, the precision of the instruments used, the degree to which they measure linearity, and the reliability with which results can be replicated. The combined uncertainty was multiplied by a coverage factor (k) of 2, and the resulting percentage was used to estimate the enlarged uncertainty at a 95% confidence level. When expressed as multiplied uncertainties, the estimated ranges for V, Co, Ni, Cd, Hg, Pd, and As were 13.3, 2.8, 16.7, 0.6, 4.1, 0.6, and 1.8. These values are all in atomic units. The results for the degree of uncertainty for each constituent in Cholic acid are presented in Table 6. Three more commercial samples of Cholic acid were also tested, and they confirmed the findings provided in Table 7.

Table 6: Uncertainty tests of sample -1

Element	Result (mg/L)	Standard deviation	Sample size	Confidence interval	Uncertainty	Results ± Uncertainty (mg/L)
V	8.967	0.285	6	95	0.232	8.967±0.232
Co	4.498	0.097	6	95	0.079	4.498±0.079
Ni	19.504	0.471	6	95	0.385	19.504±0.385
Cd	0.516	0.011	6	95	0.009	0.516±0.009
Hg	3.037	0.058	6	95	0.047	3.037±0.047
Pb	1.195	0.022	6	95	0.018	1.195±0.018
As	2.130	0.104	6	95	0.085	2.130±0.085

Table 6 presents the results of uncertainty tests for Sample-1, focusing on various elements' concentrations, standard deviations, sample sizes, confidence intervals, uncertainties, and

final results with their associated uncertainties. Each element's concentration, standard deviation, sample size, confidence interval, and uncertainty are listed in the table.

Results (mg/L) indicate the measured concentration of each element, while standard deviation quantifies the degree of variation or dispersion in the measurements. Sample size represents the number of measurements taken, and confidence intervals indicate a high degree of accuracy and reliability. Uncertainty values are relatively small, underscoring the precision of the analysis.

The final results for each element are shown in the form of a concentration range, Results Uncertainty (mg/L), within which the true value is expected to lie, given the uncertainty. In summary, the data in Table 6 indicates that the analytical methods employed are reliable and provide accurate measurements of heavy metal concentrations in the sample, which are valuable for quality control and ensuring compliance with safety standards.

Study of bias, often known as the recovery test

The recovery test included spiking levels of 1.5, 5, and 7.5 g/L for ^{111}Cd and ^{208}Pb; 4.5, 15, and 22.5 g/L for ^{75}As; 9, 30, and 45 g/L for ^{202}Hg; 15, 50, and 75 g/L for ^{59}Co; 60, 200, and 300 g/L for ^{60}Ni; and 30, 100, and 150 g/L for ^{51}V. Coefficient of variation given as RSD varied between 0.5 and 8.1%, and mean recoveries and standard deviations at different levels spanned 75.3 and 104.9. The mean recoveries and standard deviations ranged between 75.3 and 104.9, respectively. The sophisticated overview of the ICP-MS technique written by Wilschefski and Baxter is recommended for first-year students who are majoring in analytical chemistry.

Table 7: Comparison for uncertainty statistics in three different commercial samples of Cholic acid

Element	Result (mg/L)	Standard Deviation	Sample size	Confide-nce Interval	Uncertainty	Results ± Uncertainty (mg/L)
Sample-1						
V	8.967	0.285	6	95	0.232	8.967±0.232
Co	4.498	0.097	6	95	0.079	4.498±0.079
Ni	19.504	0.471	6	95	0.385	19.504±0.385
Cd	0.516	0.011	6	95	0.009	0.516±0.009

Hg	3.037	0.058	6	95	0.047	3.037±0.047
Pb	1.195	0.022	6	95	0.018	1.195±0.018
As	2.13	0.104	6	95	0.085	2.130±0.085
Sample-2						
V	9.074	0.400	6	95	0.327	9.074±0.327
Co	4.8518	0.035	6	95	0.086	4.852±0.086
Ni	20.239	0.209	6	95	0.336	20.239±0.336
Cd	0.5088	0.007	6	95	0.029	0.509±0.029
Hg	3.2107	0.051	6	95	0.037	3.211±0.037
Pb	1.2863	0.007	6	95	0.029	1.286±0.029
As	2.1908	0.022	6	95	0.014	2.191±0.014
Sample-3						
V	9.8695	0.167	6	95	0.264	9.87±0.264
Co	4.9748	0.035	6	95	0.063	4.975±0.063
Ni	19.648	0.209	6	95	0.301	19.648±0.301
Cd	0.5658	0.007	6	95	0.029	0.566±0.029
Hg	3.2527	0.051	6	95	0.021	3.253±0.021
Pb	1.3343	0.007	6	95	0.024	1.334±0.024
As	2.4686	0.022	6	95	0.086	2.469±0.086

Table 7 presents the analysis of three commercial samples of Cholic acid, each tested for heavy metal presence. Sample-1, which included elements like V, Co, Ni, Cd, Hg, Pb, and As, showed low standard deviations, indicating good precision. The confidence intervals were narrow, indicating accurate measurements. The uncertainty values were small, indicating reliability. Sample-2 and Sample-3 also showed low standard deviations, indicating precision. The final results, along with their associated uncertainties, offered vital information for quality control and safety standards compliance regarding the concentration of toxic heavy metals in the samples. The consistency in results between the three samples further strengthens the reliability and validity of the analytical process. Overall, the analytical methods used for testing Cholic acid samples are highly precise and accurate.

3.3: DISCUSSION

The discussion section interprets and provides context for the results obtained in the study, shedding light on their implications and significance. In this study, the focus was on the

elemental analysis of potentially hazardous heavy metals in Cholic acid samples using Q-ICP-MS as the analytical technique. The discussion is structured to address key aspects of the study's findings. The establishment of an acceptable internal standard is fundamental to any elemental analysis using Q-ICP-MS. The reliability of findings heavily depends on this critical step. Furthermore, the optimization of working parameters is essential for the precise and accurate measurement of elements within a sample. The results show that the chosen parameters provide high sensitivity, a wide dynamic linear range, and excellent precision. This highlights the meticulous preparation and calibration of the analytical instrument. Method validation is a crucial component in analytical chemistry, as it assesses the accuracy, precision, and reliability of the analytical procedure. In this study, the primary isotopes of several heavy metals were used to test the selectivity of the method, indicating its capability to determine specific analytes in complex matrices without interference. The validation study aimed to evaluate the method's dependability, and the results demonstrated excellent selectivity and specificity. The LOD and LOQ provide valuable information about the method's sensitivity and its ability to reliably detect and quantify analytes in a sample. The calculated LODs for the tested elements were exceptionally low, emphasizing the method's high sensitivity. This study's findings demonstrate that even trace amounts of these heavy metals can be accurately detected and quantified, a crucial aspect for environmental and safety assessments. The ability to produce results that are directly proportional to the concentration of the analyte is a key characteristic of a reliable testing method. The results of the linearity analysis, with high determination coefficients (R^2) close to 1, suggest that the method can provide accurate quantification across the specified concentration ranges. Accuracy often referred to as trueness, measures how closely the analytical procedure aligns with generally accepted true values. Precision is evaluated through parameters such as repeatability and intermediate precision. The results indicate that the method used in this study provides highly accurate and repeatable measurements. The RSD values for various elements show low levels of variability, reinforcing the precision of the method.

Uncertainty evaluation, according to EURACHEM/CITAC guidelines, provides a measure of the dispersion of values attributed to the measured. The combined uncertainty multiplied by a coverage factor is used to estimate the enlarged uncertainty at a 95% confidence level. The results show that the uncertainty values are within acceptable limits, suggesting that the measurements are reliable and precise. The recovery test assesses how well the method can detect and quantify analytes after spiking known concentrations into samples. The results

indicate that the method demonstrates good recovery for various heavy metals, suggesting that it is robust and reliable under different conditions. The comparative analysis of three different commercial samples of Cholic acid reinforced the consistency and reliability of the analytical method. The low standard deviations, narrow confidence intervals, and small uncertainties across all samples highlight the method's precision and accuracy.

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